

Na⁺/H⁺ exchanger blockade inhibits enterocyte inflammatory response and protects against colitis

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Németh, Zoltán H., Edwin A. Deitch, Csaba Szabó, Jon G. Mabley, Pál Pacher, Zoltán Fekete, Carl J. Hauser, and György Haskó. Na⁺/H⁺ exchanger blockade inhibits enterocyte inflammatory response and protects against colitis. *Am J Physiol Gastrointest Liver Physiol* 283: G122–G132, 2002. First published March 20, 2002; 10.1152/ajpgi.00015.2002.—Na⁺/H⁺ exchangers (NHEs) are integral transmembrane proteins found in all mammalian cells. There is substantial evidence indicating that NHEs regulate inflammatory processes. Because intestinal epithelial cells express a variety of NHEs, we tested the possibility that NHEs are also involved in regulation of the epithelial cell inflammatory response. In addition, since the epithelial inflammatory response is an important contributor to mucosal inflammation in inflammatory bowel disease (IBD), we examined the role of NHEs in the modulation of disease activity in a mouse model of IBD. In human gut epithelial cells, NHE inhibition using a variety of agents, including amiloride, 5-(N-methyl-N-isobutyl)amiloride, 5-(N-ethyl-N-isopropyl)-amiloride, harmaline, clonidine, and cimetidine, suppressed interleukin-8 (IL-8) production. The inhibitory effect of NHE inhibition on IL-8 was associated with a decrease in IL-8 mRNA accumulation. NHE inhibition suppressed both activation of the p42/p44 mitogen-activated protein kinase and nuclear factor- κ B. Finally, NHE inhibition ameliorated the course of IBD in dextran sulfate-treated mice. Our data demonstrate that inhibition of NHEs may be an approach worthy of pursuing for the treatment of IBD.

cytokines; lipopolysaccharide; mitogen-activated protein kinase; Crohn's disease; ulcerative colitis

THE INTESTINAL INFLAMMATION during inflammatory bowel disease (IBD) is a result of a complex interplay of immune and nonimmune cell interactions (12, 49). Emerging evidence indicates that of all the classical "nonimmune" cells present in the gut, the epithelial cells of the mucosa may play a central role in inducing and maintaining intestinal inflammation. During gut inflammation, intestinal epithelial cells (IECs) receive their activating signals from basically two sources: 1) the classical immune cells of the gut via humoral factors, such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α), and 2) direct interactions with

bacteria and bacterial products. These signals activate IECs to produce a wide range of inflammatory mediators, including the chemokine IL-8 (13, 55) and the free radical nitric oxide (28, 42, 54). Central to this inflammatory response is the transcription factor nuclear factor- κ B (NF- κ B), whose activation is responsible for the transcription of inflammatory genes in IECs during IBD (24). IL-8 is a powerful neutrophil chemoattractant and activator. Colonic IL-8 levels correlate with the macroscopic grade of local inflammation in IBD patients, in which large numbers of neutrophils are found in crypt abscesses (6, 33). In addition, IL-8 neutralization has been shown (19, 27) to abolish the neutrophil-activating effect of rectal dialysate or colonic organ cultures taken from IBD patients. Thus it appears that IL-8 of epithelial origin plays an important role in the amplification and/or maintenance of intestinal inflammation in IBD patients.

Na⁺/H⁺ exchangers (antiporters, NHE) are a family of ubiquitous plasma membrane transport proteins that catalyze the exchange of extracellular Na⁺ for intracellular H⁺ (8, 15). Recent molecular cloning studies (50, 56, 57) have confirmed that NHEs constitute a gene family from which seven mammalian isoforms (NHE1, NHE2, NHE3, NHE4, NHE5, NHE6, and NHE7) have been cloned and sequenced. IECs have been shown (22, 31, 51) to express NHE1, NHE2, NHE3, and NHE4, but the exact function of the various isoforms is unknown. The "housekeeping" NHE1 is present on the basolateral membrane, where it is involved in intracellular pH maintenance and cell volume regulation. The apically expressed NHE3 plays an important role in Na⁺, bicarbonate, and water reabsorption (7). NHEs are reversibly and selectively inhibited by the diuretic drug amiloride and its analogs as well as by a variety of nonrelated drugs, including cimetidine, clonidine, and harmaline (50, 56).

Evidence indicates that NHEs are rapidly activated in response to a variety of inflammatory signals, such as IL-1 (5), TNF- α (52), interferon- γ (IFN- γ) (41) and lipopolysaccharide (LPS) (39, 52). Conversely, NHEs have been shown to regulate the inflammatory func-

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tions of "professional" inflammatory cells, including monocytes (44), macrophages (35, 41), and neutrophils (45, 53). We hypothesized that NHEs may regulate the IEC inflammatory response because various extracellular stimuli present during gut inflammation, such as cytokines, bacteria, and bacterial products, activate NHEs and NHEs are involved in the regulation of inflammatory processes. Our results demonstrate an essential role for NHEs in mediating the IEC inflammatory response. We show that a functional NHE is required for both maximal NF- κ B activation and IL-8 production in IECs. Furthermore, we demonstrate that the NHE regulation of intestinal inflammation is also operational *in vivo*, because NHE inhibition dramatically attenuates disease activity in the mouse dextran sulfate model of IBD.

MATERIALS AND METHODS

In Vitro Studies

Cell lines. The human colon cancer cell lines HT-29 and Caco-2 were obtained from American Type Culture Collection (Manassas, VA). HT-29 cells were grown in modified McCoy's 5A medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Caco-2 cells were grown in DMEM with high glucose containing 10% fetal bovine serum (26).

Drugs and reagents. Amiloride HCl, 5-(*N*-methyl-*N*-isobutyl)amiloride (MIA), and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) were obtained from Research Biochemicals (Natick, MA). Cimetidine, harmaline, and clonidine were purchased from Sigma (St. Louis, MO). All NHE inhibitors were dissolved in DMSO. Human IL-1 β and TNF- α were obtained from R&D Systems (Minneapolis, MN). The p42/44 pathway inhibitor PD-98059 was purchased from Calbiochem (San Diego, CA). LPS (*Escherichia coli* 055:B5) and pyrrolidinedithiocarbamate were purchased from Sigma. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Fisher Scientific (Pittsburgh, PA).

IL-8 measurement. To study the effect of NHE inhibitors on IL-8 production, cells in 96-well plates were treated with IL-1 β (20 ng/ml), TNF- α (20 ng/ml), or LPS (10 μ g/ml) for 24 h. However, because harmaline was toxic to the cells when the incubation lasted for 24 h, the effect of these agents on IL-8 production was tested 4 h after IL-1 β stimulation. Furthermore, the effect of both MIA and EIPA was tested 4 h after stimulation with IL-1 β . Amiloride or its vehicle (0.5% DMSO) was added to the cells at various time points before and after stimulation with cytokines or LPS. Human IL-8 levels were determined from the cell supernatants using commercially available ELISA kits (R&D Systems), according to the manufacturer's instructions.

Western blot analysis. HT-29 cells in six-well plates were pretreated with amiloride (300 μ M) or vehicle (0.5% DMSO), and 30 min later the cells were stimulated with IL-1 β (20 ng/ml) for 15 min. After being washed with PBS, the cells were lysed by the addition of modified radioimmunoprecipitation buffer [50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.25% Na deoxycholate, 1% Nonidet P-40, 1 μ g/ml pepstatin, 1 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM Na₃VO₄]. The lysates were transferred to Eppendorf tubes and centrifuged at 15,000 *g*, and the supernatant was recovered. Protein

concentrations were determined using the Bio-Rad protein assay (Hercules, CA). Ten micrograms of sample were separated on a 8–16% Tris-glycine gel (Invitrogen) and transferred to a nitrocellulose membrane. The membranes were probed with anti-phospho-mitogen-activated protein kinase (MAPK) antibody (p42/p44; Promega, Madison, WI) and subsequently incubated with a secondary horseradish peroxidase-conjugated donkey anti-rabbit antibody (Boehringer-Mannheim, Indianapolis, IN). Bands were detected using enhanced chemiluminescence Western blotting detection reagent (Amersham Life Science, Arlington Heights, IL).

RNA isolation and RT-PCR. Total RNA was isolated from HT-29 and Caco-2 cells using TRIzol reagent (Invitrogen). RNA RT was performed using Moloney murine leukemia virus RT from Perkin-Elmer (50 U/ μ l; Foster City, CA). RNA (5 μ g) was transcribed in a 20 μ l reaction containing 10.7 μ l of RNA, 2 μ l of 10 \times PCR buffer, 2 μ l of 10 mM dNTP mix, 2 μ l of 25 mM MgCl₂, 2 μ l of 100 mM dithiothreitol (DTT), 0.5 μ l of RNase inhibitor (Perkin-Elmer, 20 U/ μ l), 0.5 μ l of 50 mM oligo(dT)₁₆, and 0.3 μ l of RT. The reaction mix was incubated at 42°C for 15 min for reverse transcription. Thereafter, the RT was inactivated at 99°C for 5 min. RT-generated DNA was amplified using Expand high-fidelity PCR system (Boehringer-Mannheim). The reaction buffer (25 μ l) contained 2 μ l of cDNA, water, 2.5 μ l of PCR buffer, 1.5 μ l of 25 mM MgCl₂, 1 μ l of 10 mM dNTP mix, 0.5 μ l of 10 μ M oligonucleotide primer (each), and 0.2 μ l enzyme. cDNA was amplified using the following primers: IL-8 (36), 5'-ATGACTTCCAAGCTGGCCGTGGCT-3' (sense) and 5'-TCTCAGCCTCTTCAAAAACCTCTC-3' (antisense); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-CGGAGTCAACGGATTTGGTCTGAT-3' (sense) and 5'-AGCCTTCTCATGGTGGTGAAGAC-3' (anti-sense). The conditions were as follows: an initial denaturation at 94°C for 5 min; 27 and 23 cycles at 94°C for 30 s for IL-8 and GAPDH, respectively; 58°C for 45 s; 72°C for 45 s; and 72°C for 7 min. The expected PCR products were 289 and 306 bp for IL-8 and GAPDH, respectively. The PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide.

NF- κ B electrophoretic mobility shift assay and supershift assay. IECs were stimulated with IL-1 β (20 ng/ml; R&D Systems) for 45 min, and nuclear protein extracts were prepared as described previously (11, 34). To determine the effect of NHE blockade, we pretreated cells with amiloride (300 μ M), harmaline (3 mM), cimetidine (6 mM), clonidine (3 mM), or vehicle (0.5% DMSO) 30 min before IL-1 β stimulation. All nuclear extraction procedures were performed on ice with ice-cold reagents. Cells were washed twice with PBS and harvested by scraping into 1 ml of PBS and pelleted at 6,000 rpm for 5 min. The pellet was resuspended in 1 packed cell volume of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl₂, 0.2% vol/vol Nonidet P-40, 1 mM DTT, and 0.1 mM PMSF) and incubated for 5 min with occasional vortexing. After centrifugation at 6,000 rpm, 1 cell pellet volume of extraction buffer (20 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl₂, 25% vol/vol glycerol, 1 mM DTT and 0.5 mM PMSF) was added to the nuclear pellet and incubated on ice for 15 min with occasional vortexing. Nuclear proteins were isolated by centrifugation at 14,000 *g* for 15 min. Protein concentrations were determined using the Bio-Rad protein assay. Nuclear extracts were stored at -70°C until used for electrophoretic mobility shift assay (EMSA). The oligonucleotide probe used for the EMSA was purchased from Promega. Oligonucleotide probes were labeled with [γ -³²P]ATP using T4 polynucle-

otide kinase (Invitrogen) and purified in Bio-Spin chromatography columns (Bio-Rad). For the EMSA analysis, 10 µg of nuclear proteins were preincubated with EMSA buffer [12 mM HEPES, pH 7.9, 4 mM Tris·HCl, pH 7.9, 25 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 50 ng/ml poly(dI-dC), 12% glycerol (vol/vol), and 0.2 mM PMSF] on ice for 10 min before the radiolabeled oligonucleotide was added for an additional 25 min. The specificities of the binding reactions were tested by incubating duplicate samples with 100-fold molar excess of the unlabeled oligonucleotide probe. Protein-nucleic acid complexes were resolved using a nondenaturing polyacrylamide gel consisting of 5% acrylamide (acrylamide/bisacrylamide, 29:1) and run in 0.5× TBE (45 mM Tris·HCl, 45 mM boric acid, and 1 mM EDTA) for 1 h at a constant current (30 mA). Gels were transferred to Whatman 3M paper, dried under vacuum at 80°C for 1 h, and exposed to photographic film at -70°C with an intensifying screen. For supershift studies, after addition of the radiolabeled probe, samples were incubated for 1 h with control, p65, or p50 antibody and then loaded on the gel.

Transient transfection and luciferase activity. For transient transfections, 2–4 × 10⁵ HT-29 cells were seeded per well of a 24-well tissue culture dish 1 day before transient transfection. Cells were transfected with 3 µl of Lipofectamine 2000 (Invitrogen) and 5–10 µg/ml of DNA containing a NF-κB luciferase promoter construct (Clontech, San Diego, CA) or the empty vector, according to the manufacturer's instructions. This NF-κB luciferase vector contains four tandem copies of the NF-κB consensus sequence fused to a TATA-like promoter region from the herpes simplex virus thymidine kinase promoter. After 5 h at 37°C, 10% fetal bovine serum was added, and cells were allowed to recover at 37°C for 20 h. The cells were pretreated with amiloride or its vehicle for 30 min and then stimulated with IL-1β for 16 h. Luciferase activity was measured by the luciferase reporter assay system (Promega) and normalized relative to micrograms of protein.

Measurement of mitochondrial respiration. Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondria-dependent reduction of MTT to formazan. Cells in 96-well plates were incubated with MTT (0.5 mg/ml) for 60 min at 37°C. The culture medium was removed by aspiration, and the cells were solubilized in DMSO (100 µl). The extent of the reduction of MTT to formazan within cells was quantitated by an optical density measurement at 550 nm using a Spectramax 250 microplate reader (17).

In Vivo Studies

Animals. Male BALB/c mice (6–8 wk of age) purchased from Taconic Farms (Germantown, NY) were used for the colitis studies. Animals were kept on a standard chow pellet diet with tap water ad libitum on a 12:12-h light-dark cycle. Animals were housed at four per cage in a room with controlled lighting (lights on from 800 to 2000) and temperature (maintained at 21–23°C) and used in experiments 3–7 days after arrival. The studies were conducted after approval of the protocols by the local institutional animal care and use committee.

Dextran sulfate-induced colitis. Colonic inflammation was induced by the administration of dextran sodium sulfate (DSS) in the drinking water (25, 30, 48). The animals were exposed to 5% DSS (molecular mass, 40–44 kDa) ad libitum. Mice were treated via one oral gavage with amiloride (10 mg·kg⁻¹·day⁻¹; gavage given twice a day with 5 mg/kg amiloride administered each time) or vehicle (saline) start-

ing on day 1 and continuing throughout the study. Water and food intake was monitored and did not differ between the various experimental groups. The parameters recorded were mortality, body weight, colon length, and myeloperoxidase (MPO) and malondialdehyde (MDA) levels. In addition, macrophage inflammatory protein-2 (MIP-2) levels were also measured in colonic homogenates using ELISA. The body weight of the animals was expressed as the mean percent decrease over the 10-day experimental period. To measure colon length, the mice were killed 10 days after the start of DSS administration. The colon was resected between the ileocecal junction and the proximal rectum, close to its passage under the pelvisternum. The dissected colon was placed on a nonabsorbent surface and measured with a ruler, taking care not to stretch the tissue. The feces was then removed from the colon using physiological buffer before the biopsies were taken and flash frozen for biochemical analysis.

The colonic levels of MPO and MDA were determined from frozen biopsies as described previously (29). MDA formation was utilized to quantify the lipid peroxidation in tissues and measured as thiobarbituric acid-reactive material. Tissues were homogenized (100 mg/ml) in 1.15% KCl buffer. The homogenates (200 µl) were then added to a reaction mixture consisting of 1.5 ml 0.8% thiobarbituric acid, 200 µl 8.1% SDS, 1.5 ml 20% acetic acid (pH 3.5), and 600 µl distilled H₂O. The mixture was then heated at 90°C for 45 min. After cooling to room temperature, the samples were cleared by centrifugation (10,000 g for 10 min), and their absorbance was measured at 532 nm, using 1,1,3,3-tetramethoxypropane as an external standard. The level of lipid peroxides was expressed as nanomoles of MDA per microgram of protein (Bradford assay). For the MPO assay, tissues were homogenized (50 mg/ml) in 0.5% hexadecyltrimethylammonium bromide in 10 mM MOPS and centrifuged at 15,000 g for 40 min. The suspension was then sonicated three times for 30 s. An aliquot of supernatant was mixed with a solution of 1.6 mM tetramethylbenzidine and 1 mM hydrogen peroxide. Activity was measured spectrophotometrically as the change in absorbance at 650 nm at 37°C, using a Spectramax microplate reader (Molecular Devices, Sunnyvale, CA). Results are expressed as milliunits of MPO activity per milligram of protein, which was determined with the Bradford assay.

Statistical evaluation. Values are expressed as mean ± SE of *n* observations. Statistical analysis of the data was performed by one-way ANOVA followed by Dunnett's test, as appropriate.

RESULTS

Inhibition of NHEs Suppresses IL-8 Production by IECs

To investigate the role of NHEs in regulating IL-8 production by epithelial cells, we first examined whether the inhibition of Na⁺/H⁺ exchange by amiloride influenced IL-8 production in the human epithelial cell line HT-29 stimulated with IL-1β. IL-1β treatment of HT-29 cells for 24 h stimulated the release of IL-8 from a baseline level of 0.5 ± 0.01 to 27.2 ± 3.1 ng/ml. Amiloride pretreatment of the cells 30 min before IL-1β stimulation caused a concentration-dependent reduction of IL-8 levels in the cell supernatants taken 24 h after the IL-1β challenge (Fig. 1A). The IC₅₀ of amiloride was ~30 µM, and the suppression amounted

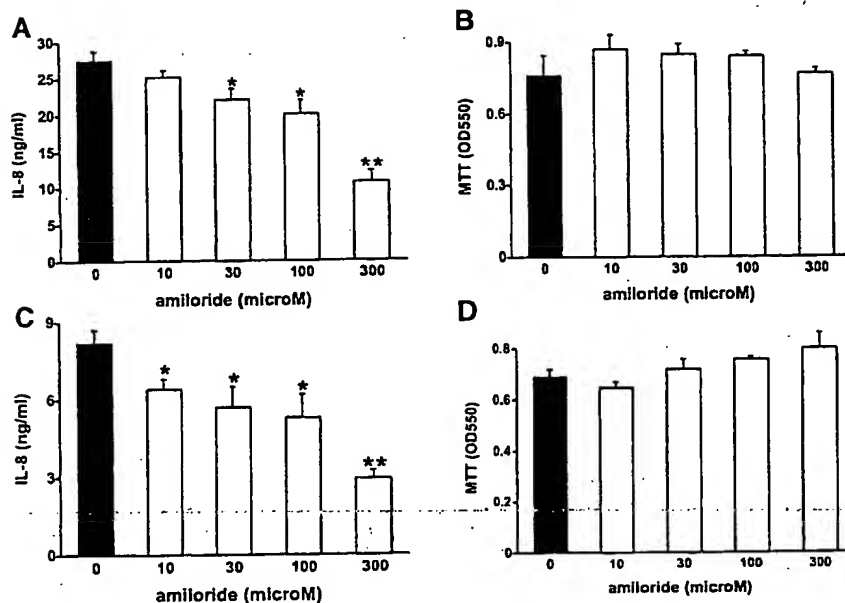


Fig. 1. Amiloride suppresses interleukin-1 β (IL-1 β)-induced IL-8 production by both HT-29 (A) and Caco-2 cells (C). Amiloride does not affect cell viability of either HT-29 (B) or Caco-2 cells (D). Amiloride was added to the cells 30 min before stimulation with IL-1 β . Supernatants for IL-8 measurement were taken 24 h after the IL-1 β challenge. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD550, optical density measurement at 550 nm. Data are means \pm SE of $n = 6$ wells. * $P < 0.05$; ** $P < 0.01$.

to ~60%. The possibility that the amiloride suppression of IL-8 production was due to a decrease in cell viability by this inhibitor was excluded using an MTT assay, which showed that amiloride did not suppress mitochondrial respiration in the 1–300 μ M concentration range (Fig. 1B). The effect of higher concentrations of amiloride on IL-8 production was not investigated, because amiloride at 1,000 μ M was toxic to the cells (data not shown).

To examine whether the effect of amiloride was cell-type specific, we investigated the effect of this agent on IL-1 β -induced IL-8 production in the Caco-2 human epithelial cell line. Amiloride suppressed IL-8 production by these cells as well (Fig. 1C), with a similar potency to that observed in HT-29 cells. This observation suggests that the reduction of IL-8 production by NHE blockade is not cell-type specific, but rather a general phenomenon. To determine whether the inhibitory effect of amiloride treatment was time dependent, we administered 300 μ M amiloride 30 min before, concurrent with, or 0.5, 1.5, 3, 4.5, 6, 7.5, or 9 h after stimulation with IL-1 β . As shown in Fig. 2, concomitant treatment with amiloride resulted in maximal suppression of IL-8 production, which amounted to $57 \pm 8.2\%$ ($n = 6$, $P < 0.01$). In addition, a significant inhibition of IL-8 production was also observed up to 4.5 h after IL-1 β stimulation. This finding demonstrates that the amiloride-induced suppression of IL-8 production does not require pretreatment.

In subsequent experiments, we examined the effect of selective inhibition of NHEs by the amiloride analogs MIA and EIPA on IL-1 β -induced IL-8 production by HT-29 cells. Figure 3 demonstrates that both MIA and EIPA suppressed IL-8 production. MIA suppressed IL-8 production with an IC₅₀ of ~10

nM (Fig. 3A). MIA was most efficacious at 100 nM; however, at higher concentrations, MIA became less effective. EIPA had a biphasic effect (Fig. 3B). The first phase of the EIPA suppression of IL-1 β -stimulated IL-8 production reached its maximum at 300 nM, followed by an elevation of IL-8 levels that reached its peak at 3 μ M EIPA. At higher EIPA concentrations, the curve showed another downward turn. However, IL-8 levels at 50 μ M EIPA were still higher than those at 300 nM. The inhibitory effect of MIA or EIPA was not due to a decrease in cell viability, because neither of these agents decreased mitochondrial respiration at the concentrations tested (data not shown).

To further corroborate a role for NHEs in the regulation of IL-8 production, we next determined the effect of a series of NHE inhibitors that are structurally unrelated to amiloride on IL-1 β -induced IL-8 produc-

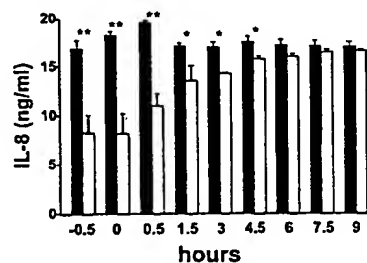


Fig. 2. Amiloride suppresses IL-1 β -induced IL-8 production in HT-29 cells in a time-dependent manner. Amiloride (300 μ M) was administered 30 min before, concurrent with, or at 0.5, 1.5, 3, 4.5, 6, 7.5, or 9 h after stimulation with IL-1 β . Supernatants for IL-8 measurement were taken 24 h after the IL-1 β challenge. Filled bars, control; open bars, amiloride. Data are means \pm SE of $n = 6$ wells. * $P < 0.05$; ** $P < 0.01$.

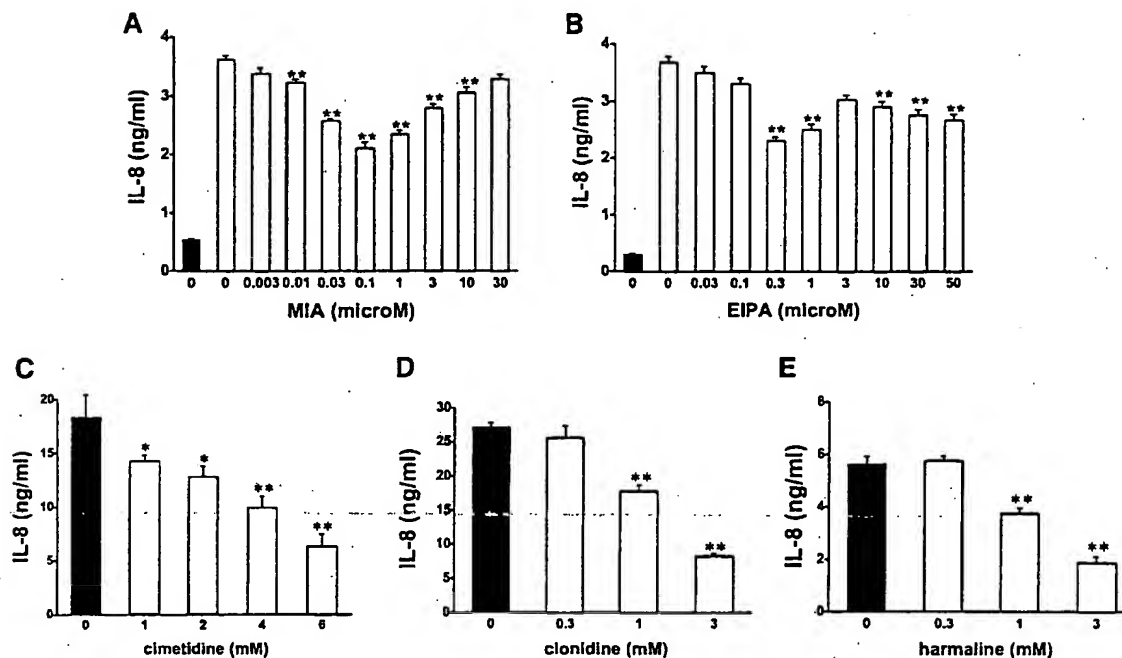


Fig. 3. A and B: treatment of HT-29 cells with the selective Na⁺/H⁺ exchanger (NHE) inhibitors 5-(N-methyl-N-isobutyl)amiloride (MIA) (A) or 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) (B) suppresses IL-1 β -induced IL-8 production. Filled bar, no IL-1 β ; open bar, IL-1 β . C–E: treatment of HT-29 cells with the nonamiloride NHE inhibitors cimetidine (C), clonidine (D), or harmaline (E) reproduces the suppressive effect of selective NHE inhibitors on the production of IL-8 by IL-1 β -induced HT-29 cells. Filled bar, vehicle, open bar, NHE inhibitor. Data are means \pm SE of $n = 12$ wells from 2 separate experiments. * $P < 0.05$, ** $P < 0.01$ compared with IL-1 β -induced values.

tion by HT-29 cells. The NHE inhibitors cimetidine, clonidine, and harmaline decreased IL-1 β -induced IL-8 production by HT-29 cells (Fig. 3, C, D, and E). The effect of all three nonamiloride NHE inhibitors was concentration dependent (Fig. 3, C, D, and E). Under the conditions studied, none of these agents decreased cell viability at the concentrations tested (data not shown). These data confirm that NHE inhibition attenuates IL-8 production by HT-29 cells.

Finally, we tested whether amiloride inhibited the production of IL-8 induced by LPS or TNF- α . Similar to our findings with IL-1 β -induced IL-8 production, amiloride pretreatment of HT-29 cells attenuated LPS- or TNF- α -induced IL-8 production (Fig. 4). In addition to suppressing cytokine- or LPS-induced IL-8 production, amiloride also decreased basal, unstimulated IL-8 production (data not shown).

Amiloride Suppresses IL-1 β -Induced IL-8 mRNA Accumulation in IECs

To determine whether the suppressive effect of NHE inhibition on IL-8 protein production was associated with an inhibitory effect on IL-8 mRNA accumulation, we examined the effect of amiloride on IL-1 β -induced accumulation of IL-8 mRNA. To this end, HT-29 cells were pretreated with amiloride (300 μ M) or its vehicle (0.5% DMSO) for 30 min, which was followed by a 3 h exposure to IL-1 β . At the end of the incubation period,

total cellular RNA was extracted and IL-8 mRNA levels were determined by semiquantitative RT-PCR. As shown in Fig. 5, the IL-1 β -induced increase in IL-8 mRNA level was prevented by pretreatment of the cells with amiloride. Amiloride also suppressed IL-1 β -induced IL-8 mRNA levels in Caco-2 cells (data not shown). The effect of amiloride was not due to a general inhibition of gene transcription, because amiloride did not affect mRNA levels of the housekeeping gene GAPDH (Fig. 5). Taken together, these data suggest that the effect of amiloride on IL-8 production is pre-translational.

NHE Blockade Inhibits IL-1 β -Induced NF- κ B Activation and Extracellular Signal-Regulated Kinase Phosphorylation

Because the effect of amiloride was pretranslational, we hypothesized that amiloride may have decreased the rate of transcription due to an effect on transcription factor activation. Because NF- κ B is one of the most important transcription factors that mediates the transcription of the IL-8 gene in response to IL-1 β , we examined whether amiloride altered the binding of NF- κ B to its consensus site using the gel mobility shift assay. Using nuclear extracts from HT-29 cells treated with IL-1 β , we observed an increase in NF- κ B binding compared with IL-1 β -untreated cells (Fig. 6, A and B). Supershift studies

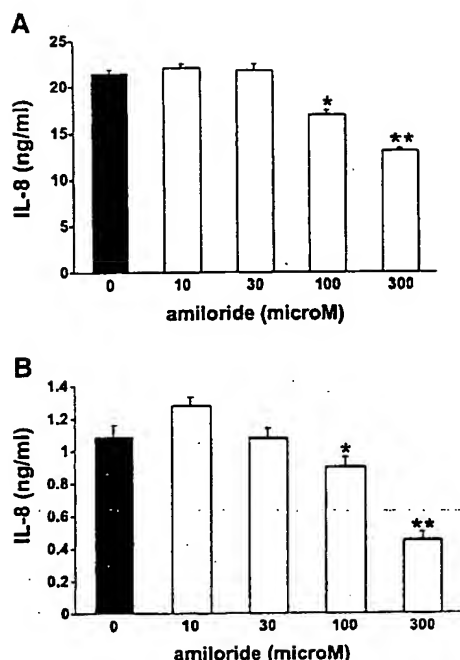


Fig. 4. Amiloride inhibits IL-8 production induced by either tumor necrosis factor- α (TNF- α) (A) or lipopolysaccharide (LPS) (B) in HT-29 cells. Amiloride was added to the cells 30 min before stimulation with TNF- α or LPS. Supernatants for IL-8 measurement were taken 24 h after stimulation. Data are means \pm SE. * P < 0.05; ** P < 0.01.

confirmed the observation by previous reports (2, 20) that the slower migrating complex induced by IL-1 β contained both p65 and p50, whereas the faster-moving, constitutive complex contained only p50. This is because both p50 and p65 antibodies completely inhibited formation of the slower moving complex, whereas the density of the faster moving complex was clearly decreased using the p50 but not the p65 antibody (Fig. 6, A and B). The increase in NF- κ B binding caused by IL-1 β stimulation was partially prevented when the cells were pretreated with amiloride 30 min before IL-1 β treatment. To further confirm that NHE inhibition attenuates NF- κ B activation, we examined the effect of harmaline, cimetidine, and clonidine on IL-1 β -induced NF- κ B DNA binding. Figure 6C demonstrates that all three NHE inhibitors suppressed NF- κ B activation. Collectively, these results suggest that the mechanism of action of NHE blockade in suppressing IL-8 production involves interference with the NF- κ B pathway.

Next, we investigated whether the decrease in NF- κ B activation corresponded with a decrease in NF- κ B-dependent gene transcription. To this end, HT-29 cells were transiently transfected with a NF- κ B-luciferase reporter construct. The transfectants were pretreated with amiloride or its vehicle for 30 min and then stimulated with IL-1 β for 16 h. The effect of amiloride on NF- κ B-dependent gene tran-

scription was assessed using the luciferase assay. Similar to its effect on NF- κ B DNA binding, amiloride suppressed IL-1 β -stimulated NF- κ B-dependent gene transcription (Fig. 7A). This effect was specific, because amiloride did not decrease luciferase activity when the cells were transfected with an enhancerless empty vector (data not shown). To further support the notion that the reduction of NF- κ B activation by NHE inhibition is an important mechanism whereby NHE inhibition reduces IL-8 production, we conducted further pharmacological experiments using the NF- κ B inhibitor pyrrolidinedithiocarbamate (59). Figure 7B shows that pyrrolidinedithiocarbamate decreased, in a concentration-dependent manner, the release of IL-8 by IL-1 β -stimulated HT-29 cells, corroborating the idea that NF- κ B is a pivotal inducer of IL-8 production in this system. Pyrrolidinedithiocarbamate was not toxic to the cells as measured using the MTT assay (data not shown).

Activation of extracellular signal-regulated kinase (ERK) 1/2 is an important step in the cascade of cellular events leading to IL-8 production in IECs (18, 23). Because amiloride has been demonstrated (3) to regulate MAPK activation, we evaluated whether the amiloride suppression of IL-8 production could be explained by an effect on this pathway. To this end, we pretreated HT-29 cells with amiloride or vehicle, and 30 min later the cells were stimulated with IL-1 β . Whole cell extracts were prepared 30 min after the IL-1 β challenge, and subsequently the activation of ERK1/2 was determined by Western blotting using an antibody against the active, phosphorylated form of ERK1/2. As shown in Fig. 8A, IL-1 β increased the activation of ERK1/2 compared with IL-1 β -untreated controls, and pretreatment of the cells with amiloride blunted this increase. Finally, to further ascertain that a decrease in ERK1/2 activation could contribute to the inhibitory effect of NHE blockade on IL-8 production, we tested whether ERK1/2 blockade by pharmacological means prevented IL-8 production by IL-1 β -induced HT-29 cells. Figure 8B demonstrates that exposure of HT-29 cells to a selective inhibitor of the

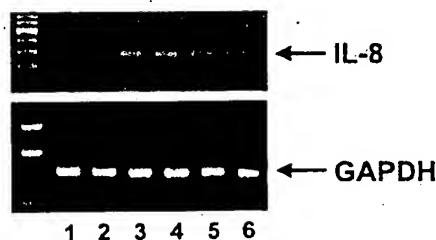
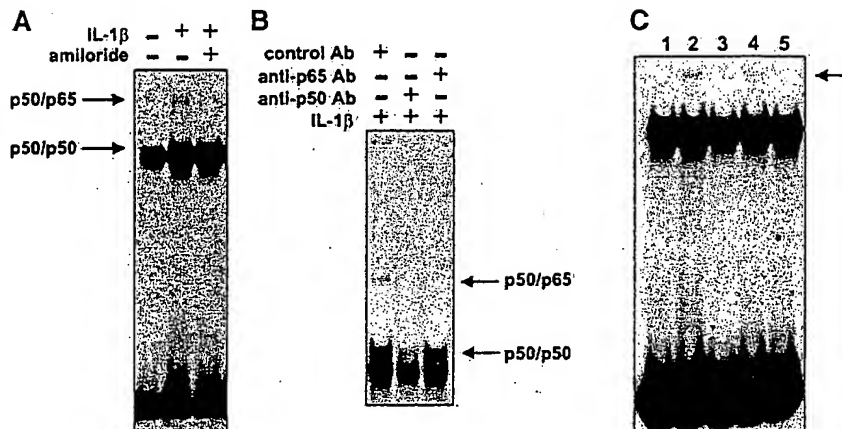


Fig. 5. Amiloride pretreatment (300 μ M) inhibits IL-1 β -induced IL-8 mRNA accumulation in HT-29 cells. Lanes 1 and 2, control; lanes 3 and 4, IL-1 β (20 ng/ml); lanes 5 and 6, amiloride + IL-1 β . Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were not affected by IL-1 β and amiloride treatment. IL-8 and GAPDH mRNA levels were quantitated using semiquantitative RT-PCR. Values represent 3 separate experiments.

Fig. 6. A and B: amiloride pretreatment (300 μ M) inhibits IL-1 β -induced nuclear factor- κ B (NF- κ B) DNA binding in HT-29 cells. NF- κ B-specific complexes are indicated by arrows as determined by antibody (Ab) supershifting. C: pretreatment of HT-29 cells with the nonamiloride NHE inhibitors cimetidine, harmaline, or clonidine reproduces the suppressive effect of amiloride IL-1 β -induced NF- κ B DNA binding in HT-29 cells. Lane 1, vehicle + no stimulus; lane 2, vehicle + IL-1 β ; lane 3, cimetidine + IL-1 β ; lane 4, clonidine + IL-1 β ; lane 5, harmaline + IL-1 β . Values represent 3 separate experiments.



ERK1/2 pathway (PD-98059) suppresses the production of IL-8. PD-98059 did not cause any toxicity as determined using the MTT assay (data not shown).

Taken together, these observations suggest that the decreased activation of ERK1/2 may represent an im-

portant mechanism by which NHE inhibition attenuates IL-8 production.

Amiloride Attenuates Course of Colitis in DSS-Treated Mice

Administration of DSS in the drinking water induced marked colonic inflammation, as evidenced by significant weight loss (Table 1), colonic shortening (Table 1), and histological injury, as well as increased MIP-2, MPO, and MDA levels in colonic samples (Fig. 9). Treatment with amiloride (10 mg/kg) induced significant protection against these alterations (Table 1 and Fig. 9).

DISCUSSION

The activation of NHEs by inflammatory stimuli crucially contributes to inflammatory gene expression in professional inflammatory cells, such as monocytes (39) and macrophages (35, 41). The increased activity of NHEs promotes several macrophage functions, including TNF- α and IL-8 production (38, 39, 44), prostaglandin release (10), upregulation of Ia expression (41), and Fc γ receptor expression (4). There is also evidence that NHE activation is involved in promoting neutrophil migration (45) as well as MPO activity and release (53). We (35) recently demonstrated that NHE inhibition by a variety of amiloride analogs suppressed IL-12, MIP-1 α , and MIP-2 production by LPS-stimulated macrophages. Furthermore, amiloride decreases IL-8 and IL-6 production in respiratory epithelium infected with respiratory syncytial virus (32). The results presented in the current study demonstrate, for the first time, that NHEs are involved in the regulation of the intestinal epithelial inflammatory response. Although amiloride is a relatively selective inhibitor of NHEs, it has effects on a number of other systems, including epithelial Na⁺ channels and the Na⁺/Ca²⁺ exchanger (21, 40, 46). However, the fact that the selective NHE inhibitors MIA (IC₅₀, ~10 nM) and EIPA (IC₅₀, ~300 nM) mimicked the inhibitory effect of amiloride on IL-8

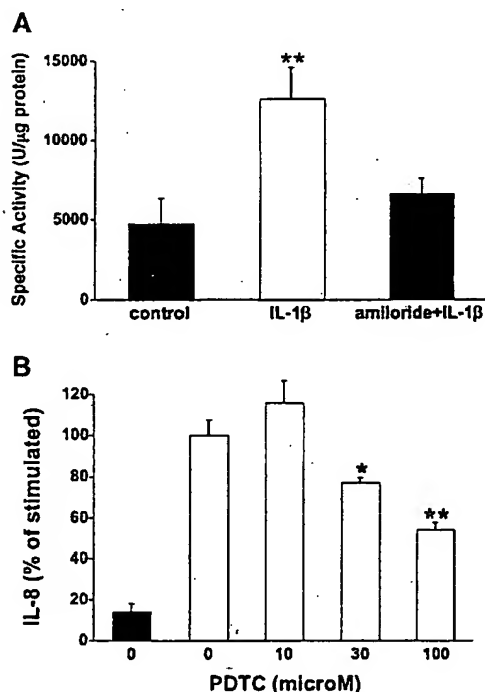


Fig. 7. A: amiloride pretreatment (300 μ M) inhibits IL-1 β -induced NF- κ B-dependent transcriptional activity in HT-29 cells transiently transfected with a NF- κ B-luciferase promoter construct. B: pretreatment of HT-29 cells with the selective NF- κ B inhibitor pyrrolidinedithiocarbamate (PDTC) 30 min before stimulation with IL-1 β (20 ng/ml) suppresses IL-1 β -induced IL-8 production. Filled bar, cells not induced with IL-1 β ; open bar, cells stimulated with IL-1 β . Data are means \pm SE of $n = 14$ –16 wells from 2 separate experiments. * $P < 0.05$; ** $P < 0.01$.

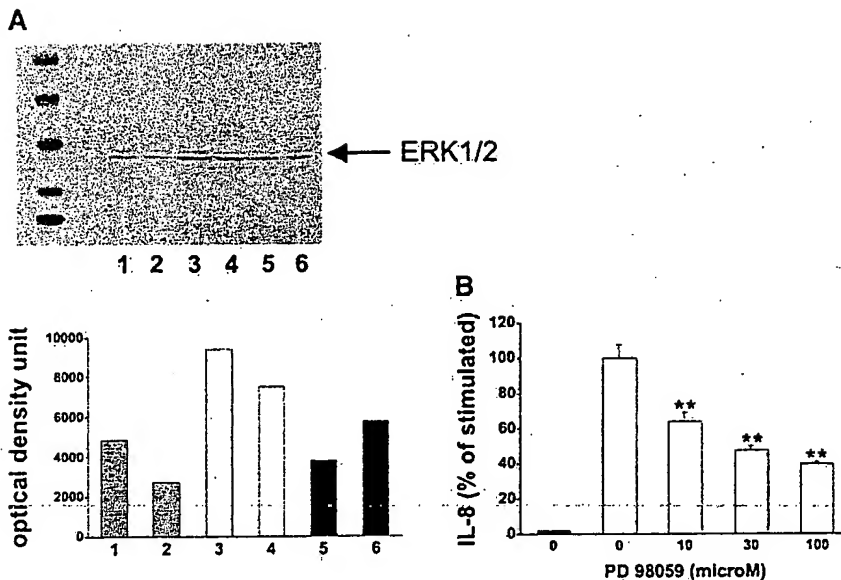


Fig. 8. A: amiloride pretreatment (300 μ M) inhibits IL-1 β -induced extracellular signal-regulated kinase (ERK) 1/2 activation in HT-29 cells. Lanes 1 and 2, control; lanes 3 and 4, IL-1 β (20 ng/ml); lanes 5 and 6, amiloride + IL-1 β . Values represent 3 separate experiments. B: pretreatment of HT-29 cells with the ERK1/2 pathway inhibitor PD-98059 30 min before stimulation with IL-1 β (20 ng/ml) suppresses IL-1 β -induced IL-8 release. Filled bar, cells not induced with IL-1 β ; open bar, cells stimulated with IL-1 β . Data are means \pm SE of $n = 14$ –16 wells from 2 separate experiments. ** $P < 0.01$.

production suggests that the primary target of amiloride in decreasing IL-8 production is the NHE. This idea was further confirmed by using the structurally different NHE inhibitors clonidine, harmaline, and cimetidine, because all three inhibitors suppressed IL-1 β -induced IL-8 production. It is important to note that we (34) recently found that lithium induced both NF- κ B activation and IL-8 production in human IECs. We (34) demonstrated that lithium, which is an agent known to stimulate NHEs, induced the intestinal epithelial inflammatory response. This lithium-induced intestinal cell inflammatory response is suppressed by the same NHE inhibitors as the IL-1 β -induced response. The potency of these inhibitors in reducing lithium-induced IL-8 production is similar to the potency of these inhibitors in decreasing IL-1 β -induced IL-8 production. Thus these data with lithium further highlight the importance of NHEs in the regulation of the IEC inflammatory response.

As described above, IECs have been shown to express NHE1, NHE2, NHE3, and NHE4; however, the expression of these isotypes varies even between different cell clones (14, 22, 31, 51). The different isotypes have differing sensitivities to inhibition by

amiloride and its analogs. At this point, it would be premature to speculate which isoforms mediate the suppressive effect of NHE inhibition on IL-8 production. The high potency of EIPA and MIA is consistent with a possible role for NHE1 or NHE2. However, this picture is complicated by the fact that amiloride was less potent in decreasing IL-8 production than would have been expected. That is, the IC₅₀ of amiloride's suppressive effect was 30 μ M, whereas amiloride has been documented to block NHE1 or NHE2 at 1 μ M (50, 56). A further complicating factor is that both MIA and EIPA lost their suppressive effects in the micromolar range. We believe that while the inhibitory effect of both MIA and EIPA in the nanomolar range reflects their ability to block NHEs, the reversal of inhibition in the micromolar range may be a nonspecific action. In any case, further studies are needed to characterize the NHE isoform(s) involved in the regulation of epithelial cell inflammatory responses.

Our data documenting that amiloride suppresses IL-8 mRNA accumulation are in agreement with the results of previous studies, which showed that amiloride suppressed IL-8 mRNA accumulation in human

Table 1. Effect of amiloride on acute colon inflammation induced by DSS in male BALB/c mice

Groups	Decrease in Body Weight, %	Colon Length, cm	Gross Score (median)	Bleeding Incidence, %
Sham	-7.0 \pm 3.1	6.1 \pm 0.3	0	0
DSS + vehicle	27.1 \pm 2.0*	3.5 \pm 0.2*	2.5*	100*
DSS + amiloride	10.4 \pm 3.1*†	5.1 \pm 0.3*†	1*†	25*†

Values are means \pm SE of $n = 8$ animals/group. Animals were initially weighed and body weights recorded before being exposed to the 5% dextran sodium sulfate (DSS) solution ad libitum. Amiloride (10 mg/kg⁻¹/day⁻¹) was administered orally BID starting on day 1. On day 10, the experiment was terminated and the animals reweighed and killed. The colon was dissected out and measured before samples were taken and analyzed for biochemical changes. The median gross score is given. * $P < 0.05$, DSS animals vs. untreated animals; † $P < 0.05$, amiloride-treated DSS animals vs. vehicle-treated DSS animals.

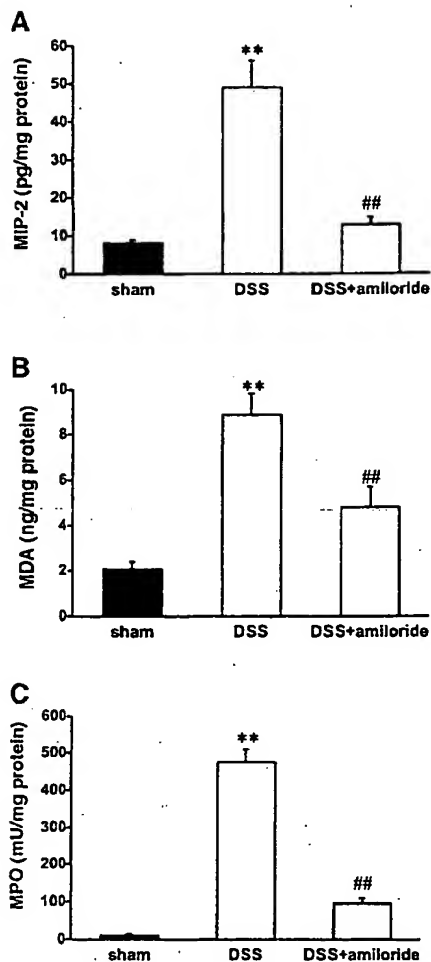


Fig. 9. Effect of amiloride in the dextran sodium sulfate (DSS) colitis model: reduction in macrophage inflammatory protein-2 (MIP-2) production, lipid peroxidation, and neutrophil infiltration. Amiloride suppressed MIP-2 (A), malondialdehyde (MDA) (B), and myeloperoxidase (MPO) (C) levels in the colon of mice with an acute inflammation induced by DSS. Mice were exposed to DSS ad libitum for 10 days; treatment with amiloride (10 mg·kg⁻¹·day⁻¹, BID) commenced on day 1. On day 10, the colon was removed and biopsies taken for determination of MIP-2, MDA, and MPO levels. Data are means ± SE from *n* = 8 animals/group. ***P* < 0.01, DSS animals vs. sham animals; ##*P* < 0.01, amiloride-treated DSS animals vs. vehicle-treated DSS animals.

monocytes (44) or respiratory epithelium (32). Interestingly, in addition to demonstrating that amiloride suppresses IL-8 production, both of these previous studies (32, 44) found that amiloride attenuated the production of other inflammatory cytokines, including IL-6 and TNF- α . This observation and the fact that the effect of amiloride on cytokine production is likely to be transcriptional suggest that amiloride targets an early pathway that is common to the induction of proinflammatory cytokines. Our results demonstrating that amiloride, harmaline, cimetidine, and clonidine prevent both NF- κ B DNA bind-

ing and NF- κ B-dependent transcriptional activity in IECs indicate that this transcription factor may be the primary mediator of the anti-inflammatory effects of NHE inhibition. This notion is supported by the findings of a recent study (16), in which amiloride blocked NF- κ B activation in respiratory epithelial cells.

These findings raise an important question: how is a signal provided by the membrane protein NHE transmitted to the cytosolic protein NF- κ B? One of the most intriguing possibilities is that it is the alteration of the cytoskeletal organization of actin filaments that links NHEs to NF- κ B and MAPK activation. This possibility is supported by the fact that NHEs are important regulators of actin filament assembly (9, 47) and that changes in the actin microfilament system are involved in the activation of the NF- κ B system (1, 58). Clearly, further studies will be necessary to delineate the mechanisms whereby NHEs couple extracellular inflammatory signals to activation of the intracellular inflammatory cascade and NF- κ B.

We also provide evidence that NHE inhibition by amiloride suppresses disease activity in the mouse DSS model of IBD. This decrease in IBD activity was associated with a decrease in MIP-2 production in colonic samples. MIP-2 or its human analog IL-8 is released from numerous sources during IBD. These include IECs as well as monocytes/macrophages (12, 49). Because we have shown that NHE inhibition suppresses MIP-2/IL-8 production by both epithelial cells (the current study) and macrophages (35), we propose that NHE inhibition exerts its beneficial effect, at least in part, by inhibiting the production of MIP-2/IL-8 by a variety of inflammatory cell types. Furthermore, we speculate that the mechanism of NHE promotion of inflammatory processes may have evolved as a positive feedback signal during inflammatory cell activation, and dysregulation of NHE activation may contribute to the maintenance of inflammatory processes during inflammatory/autoimmune diseases. On the other hand, recent evidence (43) suggests that NHEs and the intestinal epithelial inflammatory response are interconnected on yet another level. That is, an interesting study by Rocha et al. (43) showed that chronic treatment with the inflammatory cytokine IFN- γ downregulated the expression of NHEs in IECs. Thus it can be proposed that the downregulation of NHE expression by inflammatory signals may serve as a protective mechanism against the NHE-amplified inflammatory response during chronic inflammatory states.

In summary, our data demonstrate that NHE inhibition has anti-inflammatory effects in the gut, suggesting that the inhibition of Na⁺/H⁺ exchange may be a therapeutic approach in IBD.

REFERENCES

1. Are AF, Galkin VE, Pospelova TV, and Pinaev GP. The p65/RelA subunit of NF- κ B interacts with actin-containing structures. *Exp Cell Res* 256: 533–544, 2000.
2. Böcker U, Schottelius A, Watson JM, Holt L, Licato LL, Brenner DA, Sartor RB, and Jobin C. Cellular differentiation causes a selective down-regulation of interleukin (IL)-1 β -medi-

- ated NF- κ B activation and IL-8 gene expression in intestinal epithelial cells. *J Biol Chem* 275: 12207–12213, 2000.
3. Callus B, Tilbrook PA, Busfield SJ, Cull VS, Bittorf T, and Klinken SP. Amiloride suppresses erythropoietin-induced proliferation and MAP kinase, but potentiates differentiation of J2E cells. *Exp Cell Res* 219: 39–46, 1995.
4. Cassatella MA, Flynn RM, Amezcua MA, Bazzoni F, Vicentini F, and Trinchieri G. Interferon gamma induces in human neutrophils and macrophages expression of the mRNA for the high affinity receptor for monomeric IgG (Fc gamma R-1 or CD64). *Biochem Biophys Res Commun* 170: 582–588, 1990.
5. Civitelli R, Teitelbaum SL, Hruska KA, and Lacey DL. IL-1 activates the Na⁺/H⁺ antiporter in a murine T cell. *J Immunol* 143: 4000–4008, 1989.
6. Daig R, Andus T, Aschenbrenner E, Falk W, Scholmerich J, and Gross V. Increased interleukin 8 expression in the colon mucosa of patients with inflammatory bowel disease. *Gut* 38: 216–222, 1996.
7. Daniel H. Function and molecular structure of brush border membrane peptide/H⁺ symporters. *J Membr Biol* 154: 197–203, 1996.
8. Demaurex N and Grinstein S. Na⁺/H⁺ antiporter: modulation by ATP and role in cell volume regulation. *J Exp Biol* 196: 389–404, 1994.
9. Denker SP, Huang DC, Orlowski J, Furthmayr H, Barber DL. Direct binding of the Na/H exchanger NHE1 to ERM proteins regulates the cortical cytoskeleton and cell shape independently of H⁺ translocation. *Mol Cell* 6: 1425–1436, 2000.
10. Dieter P, Schulze-Specking A, Karck U, and Decker K. Prostaglandin release but not superoxide production by rat Kupffer cells stimulated in vitro depends on Na⁺/H⁺ exchange. *Eur J Biochem* 170: 201–206, 1987.
11. Eaves-Pyles T, Szabó C, and Salzman AL. Bacterial invasion is not required for activation of NF- κ B in enterocytes. *Infect Immun* 67: 800–804, 1999.
12. Fiocchi C. Intestinal inflammation: a complex interplay of immune and nonimmune cell interactions. *Am J Physiol Gastrointest Liver Physiol* 273: G769–G775, 1997.
13. Gewirtz AT, Simon PO Jr, Schmitt CK, Taylor LJ, Hagedorn CH, O'Brien AD, Neish AS, and Madara JL. *Salmonella typhimurium* translocates flagellin across intestinal epithelia, inducing a proinflammatory response. *J Clin Invest* 107: 99–109, 2001.
14. Gonda T, Maouyo D, Rees SE, and Montrose MH. Regulation of intracellular pH gradients by identified Na/H exchanger isoforms and a short-chain fatty acid. *Am J Physiol Gastrointest Liver Physiol* 276: G259–G270, 1999.
15. Grinstein S and Wiczkorek H. Cation antiports of animal plasma membranes. *J Exp Biol* 196: 307–318, 1994.
16. Haddad JJ and Land SC. Amiloride blockades lipopolysaccharide-induced proinflammatory cytokine biosynthesis in an IkB- α /NF- κ B-dependent mechanism. Evidence for the amplification of an anti-inflammatory pathway in the alveolar epithelium. *Am J Respir Cell Mol Biol* 26: 114–126, 2002.
17. Haskó G, Szabó C, Németh ZH, Kvetan V, Pastores SM, and Vizi ES. Adenosine receptor agonists differentially regulate IL-10, TNF- α , and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice. *J Immunol* 157: 4634–4640, 1996.
18. Hobbie S, Chen LM, Davis RJ, and Galan JE. Involvement of mitogen-activated protein kinase pathways in the nuclear responses and cytokine production induced by *Salmonella typhimurium* in cultured intestinal epithelial cells. *J Immunol* 159: 5550–5559, 1997.
19. Ina K, Kusugami K, Yamaguchi T, Imada A, Hosokawa T, Ohsuga M, Shinoda M, Ando T, Ito K, and Yokoyama Y. Mucosal interleukin-8 is involved in neutrophil migration and binding to extracellular matrix in inflammatory bowel disease. *Am J Gastroenterol* 92: 1342–1346, 1997.
20. Inan MS, Rasoulopour RJ, Yin L, Hubbard AK, Rosenberg DW, and Giardina C. The luminal short-chain fatty acid butyrate modulates NF- κ B activity in a human colonic epithelial cell line. *Gastroenterology* 118: 724–734, 2000.
21. Ismailov II, Kieber-Emmons T, Lin C, Berdiev BK, Shlyonsky VG, Patton HK, Fuller CM, Worrell R, Zuckerman JB, Sun W, Eaton DC, Benos DJ, and Kleyman TR. Identification of an amiloride binding domain within the α -subunit of the epithelial Na⁺ channel. *J Biol Chem* 272: 21075–21083, 1997.
22. Janecki AJ, Montrose MH, Tse CM, de Medina FS, Zweibaum A, and Donowitz M. Development of an endogenous epithelial Na⁺/H⁺ exchanger (NHE3) in three clones of caco-2 cells. *Am J Physiol Gastrointest Liver Physiol* 277: G302–G305, 1999.
23. Jobin C, Bradham CA, Russo MP, Juma B, Narula AS, Brenner DA, and Sartor RB. Curcumin blocks cytokine-mediated NF- κ B activation and proinflammatory gene expression by inhibiting inhibitory factor I- κ B kinase activity. *J Immunol* 163: 3474–3483, 1999.
24. Jobin C and Sartor RB. NF- κ B signaling proteins as therapeutic targets for inflammatory bowel diseases. *Inflamm Bowel Dis* 6: 206–213, 2000.
25. Kato S, Hokari R, Matsuzaki K, Iwai A, Kawaguchi A, Nagao S, Miyahara T, Itoh K, Ishii H, and Miura S. Amelioration of murine experimental colitis by inhibition of mucosal addressin cell adhesion molecule-1. *J Pharmacol Exp Ther* 295: 183–189, 2000.
26. Kennedy M, Denenberg AG, Szabó C, and Salzman AL. Poly(ADP-ribose) synthetase activation mediates increased permeability induced by peroxynitrite in Caco-2BBE cells. *Gastroenterology* 114: 510–518, 1998.
27. Keshavarzian A, Fusunyan RD, Jacyno M, Winship D, MacDermott RP, Sanderson IR. Increased interleukin-8 (IL-8) in rectal dialysate from patients with ulcerative colitis: evidence for a biological role for IL-8 in inflammation of the colon. *Am J Gastroenterol* 94: 704–712, 1999.
28. Kolios G, Rooney N, Murphy CT, Robertson DA, and Westwick J. Expression of inducible nitric oxide synthase activity in human colon epithelial cells: modulation by T lymphocyte-derived cytokines. *Gut* 43: 56–63, 1998.
29. Liaudet L, Soriano FG, Szabó E, Virág L, Mabley JG, Salzman AL, and Szabó C. Protection against hemorrhagic shock in mice genetically deficient in poly(ADP-ribose) polymerase. *Proc Natl Acad Sci USA* 97: 10203–10208, 2000.
30. Mabley JG, Jagtap P, Perretti M, Getting SJ, Salzman AL, Virág L, Szabó E, Soriano FG, Liaudet L, Abdelkarim GE, Haskó G, Marton A, Southan GJ, and Szabó C. Anti-inflammatory effects of a novel, potent inhibitor of poly (ADP-ribose) polymerase. *Inflamm Res* 50: 561–569, 2001.
31. Maouyo D, Chu S, and Montrose MH. pH heterogeneity at intracellular and extracellular plasma membrane sites in HT29-C1 cell monolayers. *Am J Physiol Cell Physiol* 278: C973–C981, 2000.
32. Mastrorade JG, Monick MM, Gross TJ, and Hunninghake GW. Amiloride inhibits cytokine production in epithelium infected with respiratory syncytial virus. *Am J Physiol Lung Cell Mol Physiol* 271: L201–L207, 1996.
33. Mazzucchielli L, Hauser C, Zraggen K, Wagner H, Hess M, Laissue JA, and Mueller C. Expression of interleukin-8 gene in inflammatory bowel disease is related to the histological grade of active inflammation. *Am J Pathol* 144: 997–1007, 1994.
34. Németh ZH, Deitch EA, Szabó C, Fekete Z, Hauser CJ, Haskó G. Lithium induces NF- κ B activation and interleukin-8 production in human intestinal epithelial cells. *J Biol Chem* 277: 7713–7719, 2002.
35. Németh ZH, Deitch EA, Szabó C, and Haskó G. Inhibition of the Na⁺/H⁺ antiporter suppresses IL-12 p40 production by mouse macrophages. *Biochim Biophys Acta* 1539: 233–242, 2001.
36. Nilsen EM, Johansen FE, Jahnsen FL, Lundin KEA, Scholz T, Brandtzaeg P, and Haraldsen G. Cytokine profiles of cultured microvascular endothelial cells from the human intestine. *Gut* 42: 635–642, 1998.
37. Numata M and Orlowski J. Molecular cloning and characterization of a novel (Na⁺, K⁺)/H⁺ exchanger localized to the trans-Golgi network. *J Biol Chem* 276: 17387–17394, 2001.
38. Ohmori Y, Reynolds E, and Hamilton TA. Modulation of Na⁺/K⁺ exchange potentiates lipopolysaccharide-induced gene

- expression in murine peritoneal macrophages. *J Cell Physiol* 148: 96–105, 1991.
39. Orlinska U and Newton RC. Modification of tumor necrosis factor- α (TNF- α) production by the Na⁺-dependent HCO₃⁻ cotransport in lipopolysaccharide-activated human monocytes. *Immunopharmacology* 30: 41–50, 1995.
 40. Philipson KD and Nicoll DA. Sodium-calcium exchange: a molecular perspective. *Annu Rev Physiol* 62: 111–133, 2000.
 41. Prpic V, Yu SF, Figueiredo F, Hollenbach PW, Gawdi G, Herman B, Uhing RJ, and Adams DO. Role of Na⁺/H⁺ exchange by interferon- γ in enhanced expression of JE and I-A beta genes. *Science* 244: 469–471, 1989.
 42. Rachmilewitz D, Stamler JS, Bachwich D, Karmeli F, Ackerman Z, and Podolsky DK. Enhanced colonic nitric oxide generation and nitric oxide synthase activity in ulcerative colitis and Crohn's disease. *Gut* 36: 718–723, 1995.
 43. Rocha F, Musch MW, Lishansky L, Bookstein C, Sugi K, Xie Y, and Chang EB. IFN- γ downregulates expression of Na⁺/H⁺ exchangers NHE2 and NHE3 in rat intestine and human Caco-2/bbe. *Am J Physiol Cell Physiol* 280: C1224–C1232, 2001.
 44. Rolfe MW, Kunkel SL, Rowens B, Standiford TJ, Cragoe EJ Jr, and Strieter RM. Suppression of human alveolar macrophage-derived cytokines by amiloride. *Am J Respir Cell Mol Biol* 6: 576–582, 1992.
 45. Rosengren S, Henson PM, and Worthen GS. Migration-associated volume changes in neutrophils facilitate the migratory process in vitro. *Am J Physiol Cell Physiol* 267: C1623–C1632, 1994.
 46. Schild L, Schneeberger E, Gautschi I, and Firsov D. Identification of amino acid residues in the alpha, beta, and gamma subunits of the epithelial sodium channel (ENaC) involved in amiloride block and ion permeation. *J Gen Physiol* 109: 15–26, 1997.
 47. Shenolikar S and Weinman EJ. NHERF: targeting and trafficking membrane proteins. *Am J Physiol Renal Physiol* 280: F389–F395, 2001.
 48. Siegmund B, Rieder F, Albrich S, Wolf K, Bidlingmaier C, Firestein GS, Boyle D, Lehr HA, Locher F, Hartmann G, Endres S, and Eigler A. Adenosine kinase inhibitor GP515 improves experimental colitis in mice. *J Pharmacol Exp Ther* 296: 99–105, 2001.
 49. Strober W. Interactions between epithelial cells and immune cells in the intestine. *Ann NY Acad Sci* 859: 37–45, 1998.
 50. Szabo EZ, Numata M, Shull GE, and Orlowski J. Kinetic and pharmacological properties of human brain Na⁺/H⁺ exchanger isoform 5 stably expressed in Chinese hamster ovary cells. *J Biol Chem* 275: 6302–6307, 2000.
 51. Turner JR, Black ED, Ward J, Tse CM, Uchwat FA, Alli HA, Donowitz M, Madara JL, and Angle JM. Transepithelial resistance can be regulated by the intestinal brush-border Na⁺/H⁺ exchanger NHE3. *Am J Physiol Cell Physiol* 279: C1918–C1924, 2000.
 52. Vairo G, Royston AK, and Hamilton JA. Biochemical events accompanying macrophage activation and the inhibition of colony-stimulating factor-1-induced macrophage proliferation by tumor necrosis factor- α , interferon- γ , and lipopolysaccharide. *J Cell Physiol* 151: 630–641, 1992.
 53. Witko-Sarsat V, Allen RC, Paulais M, Nguyen AT, Bessou G, Lenoir G, and Descamps-Latscha B. Disturbed myeloperoxidase-dependent activity of neutrophils in cystic fibrosis homozygotes and heterozygotes, and its correction by amiloride. *J Immunol* 157: 2728–2735, 1996.
 54. Witthoft T, Eckmann L, Kim JM, and Kagnoff MF. Enteroinvasive bacteria directly activate expression of iNOS and NO production in human colon epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 275: G564–G571, 1998.
 55. Yang SK, Eckmann L, Panja A, and Kagnoff MF. Differential and regulated expression of C-X-C, C-C, and C-chemokines by human colon epithelial cells. *Gastroenterology* 113: 1214–1223, 1997.
 56. Yu FH, Shull GE, and Orlowski J. Functional properties of the rat Na/H exchanger NHE-2 isoform expressed in Na/H exchanger-deficient Chinese hamster ovary cells. *J Biol Chem* 268: 25536–25541, 1993.
 57. Yun CH, Tse CM, Nath SK, Levine SA, Brant SR, and Donowitz M. Mammalian NHE gene family: structure and function studies. *Am J Physiol Gastrointest Liver Physiol* 269: G1–G11, 1995.
 58. Zhu P, Xiong W, Rodgers G, and Qvarnstrom EE. Regulation of interleukin 1 signalling through integrin binding and actin reorganization: disparate effects on NF- κ B and stress kinase pathways. *Biochem J* 330: 975–981, 1998.
 59. Zouki C, Jozsef L, Ouellet S, Paquette Y, and Filep JG. Peroxynitrite mediates cytokine-induced IL-8 gene expression and production by human leukocytes. *J Leukoc Biol* 69: 815–824, 2001.

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